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The regulatory role of Nrf2 in antioxidants phase2 enzymes and IL-17A expression in patients with ulcerative colitis

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ABSTRACT

Background: Reactive oxygen species (ROS) is one of the pathogenic factors responsible for intestinal injury in Ulcerative colitis (UC). Nuclear factor erythroid-2 related factor 2 (Nrf2) plays a critical role against ROS factors to conserve epithelial integrity. This study aimed to localize Nrf2 and IL-17A protein in the inflamed mucosa of patients with ulcerative colitis. The gene expression of Nrf2 was also correlated with GST-A4 and PRDX1.

Materials and methods: A total of 20 patients and 20 healthy controls with definite UC based on the clinical criteria were enrolled for this study. The expression pattern of Nrf2 and IL-17A protein was compared in inflamed and non-inflamed colonic biopsies by immunohistochemical staining. *Nrf2*, *GST-A4* and *PRDX1* gene expression were determined by real-time polymerase chain reaction (RT-PCR).

Results: In inflamed colonic biopsies, an increased level of Nrf2 protein factor was detected in epithelial cells. Conversely, IL-17A protein was presented more in mononuclear cells in mucosa and lamina propria regions. A significant increase of *Nrf2*, *GST-A4* gene expression was observed in both mild and severe patients with ulcerative colitis. *GST-A4* gene expression indicated a high exponential rate in logistic regression.

Conclusion: Oxidative stress in inflamed colonic tissue can induce *Nrf2* gene expression. The performance of Nrf2 transcription factor may lead to the induction of GST-A4 and PRDX1. IL-17A is less detected in intestinal inflammation, presenting Nrf2 factor. The present findings suggest that Nrf2 function in the gut plays a role in arresting both inflammatory response and oxidative damages of UC.

1. Introduction

The inflammatory bowel diseases (IBDs) has a wide range of associated disorders like ulcerative colitis (UC), characterised by recurrent chronic and incurable inflammatory conditions in the colon and rectum [1,2]. The interaction between genetic, nutritional and environmental agents initially presents more of the inflammatory conditions in UC [3]. Potentially, Inflammation damages the mucosal barrier function and amplify inflammatory response in the gastrointestinal tract [4]. Recent investigations have also indicated an association to exist between the degree of inflammation and the enhanced production of reactive oxygen species (ROS) such as nitric oxide and superoxide [5]. Under oxidative stress, nuclear factor erythroid 2 related factor 2 (Nrf2) can be released from Keap1 and translocates into the nucleus of cells [6]. The Nrf2 is mainly expressed in the nucleus of epithelial cells to conserve the intestinal integrity. The protective effects of Nrf2 in a chronic colitis mice caused lower expression of inflammatory cytokines [7]. Activated Nrf2 has the potential to suppress interleukin (IL)-17 expression in autoimmune disease [8]. In murine dextran sodium sulfate (DSS)-induced colitis, IL-17 augments inflamed intestinal by pro-inflammatory

Abbreviations: ROS, reactive oxygen species; UC, ulcerative colitis; Nrf2, nuclear factor erythroid 2 related factor 2; IBD, inflammatory bowel diseases; DSS, dextran sodium sulfate; IL-17, interleukin-17; APE1, apurinic/apyrimidinic endonuclease; PRDX1, peroxiredoxin-1; GST-A4, glutathione *S*-transferase-alpha4; 4-HNE, trans-4-hydroxy-2-nonenal; IEC, intestinal epithelial cells; COPD, chronic obstructive pulmonary disease; Hmox-1, heme oxygenase-1; RORct, retinoic orphan receptor gamma(t); MAPK, mitogen-activated protein kinase

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effects on epithelium [9]. In addition to high level of IL-17 in serum and tissue, the existence of T helper-17 illustrates the pathogenesis role of this cytokine in the UC patients (Graphical abstract) [10,11].

The Nrf2 factor controls the expression of phase2 antioxidant enzymes by binding to the Antioxidant Response Element (ARE) region [12]. Peroxiredoxin-1 (PRDX1), a cytoprotective gene, is induced by Nrf2 activation under oxidative stress condition to protect the cell particularly against superoxide. PRDX1 can act as chaperone, protecting protein factors through thiol-specific groups in the cytoplasm [13]. PRDX1 enzyme engages in several redox factors like apurinic/apyrimidinic endonuclease1 (APE1), consequently causing a reduction in inflammatory cytokines [14]. Nrf2 can also target ARE region to induce Glutathione S-transferase alpha 4 (GST-A4) against oxidative damage. In response to accumulation of lipid peroxidation, GST-A4 attenuates toxic lipid aldehydes by conjugating to glutathione [15]. GST-A4 is dominantly capable of detoxifying trans-4-hydroxy-2-nonenal (4-HNE) which is considered as an endogenous mutagen in inflammation-associated colorectal cancer [16].

In this study, the *Nrf2* gene expression and its relation with mRNA level of PRDX1 and GST-A4 in control colonic biopsy compared to UC patient, will be examined. The expression pattern of the Nrf2 and IL-17A protein in histological tissues will be assessed. A negative protein expression between Nrf2 and IL-17A will be identified. Moreover, Nrf2 factor is more presented in mucosa layer of biopsies. These data suggest that GST-A4 gene expression appears to be dependent on Nrf2 transcription factors.

2. Materials and methods

2.1. Sample collection and preparation

The study group comprised of 20 patients with UC and 21 healthy individuals as control (Table 1). Over the period of 6 months in 2017, all cases were attended in Gastroenterology Unit of Hajar hospital, Shahrekord, Iran, from January to May 2017. Prior to the colonoscopy procedure, clinical information of UC patients such as age, gender, food habits, stress, medicine consumption and history of abdominal surgery were collected by opt query and medical files. The disease activity index (DAI) of ulcerative colitis was marked in regard to the weight loss, blood in the stool and stool consistency [17]. In addition to the clinical criteria, inflammation extension in colonoscopy and histological manifests were analyzed based on the Montreal Classification for the confirmation of UC [18]. 14 active UC patients had a mild proctosigmoiditis (inflammation was limited from the colorectum distal to the splenic flexure/erythema is detected in rectum and sigmoid, decreased friability and vascular pattern/Montreal class: E2). Six severe patients had consumed corticosteroid six months before sampling date (Involvement extends proximal to the splenic flexure/Montreal class: E3). The Control group was selected among healthy individuals without immune-mediated diseases. These subjects were undergone colonoscopy due to screening for colorectal cancer or polyp surveillance without inflammatory disease or any medication. This project was approved by Shahrekord University of Medical Sciences Ethics Committee, and all informed participants complemented a written consent.

Table 1
demographic information of the patients.

	Control	Mild colitis	Severe colitis	P value
Age	33.81 ± 8.85	35.43 ± 9.55	32.00 ± 9.06	0.729
Gender (male/female)	12/9	7/7	3/3	0.913
Smoking	9.5%	21.4%	46.7%	**0.019**[a]
Habitat (rural/urban)	2/19	4/10	3/3	0.078
History of abdominal surgery	19%	64.3%	50%	0.295

[a] Number of smoking people were statistically significance.

2.2. RNA isolation and quantitative RT-PCR

The total RNA was extracted from nitrogen frozen colon biopsies with TRIzol® reagent (Invitrogen/Thermo Fisher Scientific, Inc, catalog number. 15596026). The concentration of each RNA samples was determined by Thermo Scientific™ NanoDrop 2000. The 260/280 - 260/230 ratios for samples were measured, and cDNAs were synthesized using the Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, K1622) with 1.5 µg of RNA in a reaction volume of 20 µL. To eliminate possible DNA contamination, DNaseI (Fermentas EN0521) enzyme was used. The real-time RT-PCR to indicate the quantification of mRNA was performed on a Rotor-Gene RG-300 (Corbett Research, Sydney, AU) and SYBR Green Real-time PCR Master Mix Kit (TAKARA, Japan, catalog number. RR820Q) according to the protocol provided by the manufacturer. Then, three pairs primers were exploited for the specific amplification of Nrf2, PRDX1 and GST-A4 (Supplement File 1). The primers were designed by Primer3.0 (http://bioinfo.ut.ee/primer3-0.4.0) web-based server. We checked out the lack of SNPs in the genomic region corresponding to the 3′ ends of primers by looking through the dbSNP database. The primers specificity was checked by the in-silico-PCR tool in UCSC genome browser and Primer blast of NCBI genome browser. Thermal cycling for running a process started the denaturation step at 95 °C for 5 min first denaturation step at 95 C for 5 min and followed by 38 cycles of 95 °C for 15 s, 61 °C for 20 and 72 for 25 s. Melting curve confirmed the amplification specificity of three gene expression of Nrf2, PRDX1 and GST-A4 were normalised versus GAPDH as an internal control and relative quantification ($2-\Delta\Delta Cq$) showed the fold changes of each mRNA.

2.3. Immunohistochemistry

Immunohistochemical staining was implemented with the streptavidin biotin peroxidase-complex method according to the Abcam protocol [[19]]. Briefly, sections of biopsy specimens were cut into 4-µm-thick sections and stuck on poly-L-lysine slides. The sections were deparaffinized and rehydrated after placed in xylene and a series of alcohols (100%, 100%, 80% and 70%). In antigen retrieving stage, sections immersed in citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) were exposed to pressure for 20 min. Sections were then incubated with protein block (Abcam, England) containing albumin for 120 min to prevent nonspecific background staining. Rabbit anti-human Nrf2 antibody (ab31163, Abcam, UK) at a 1:700 dilution and anti-IL17A antibody (ab79056) were incubated overnight 4 C. It is followed by adding 0.3% H_2O_2 solved in TBS to inhibit endogenous peroxidase activity. After incubating biotinylated IgG antibody (ab93697, Abcam, UK) and Streptavidin-Peroxidase Plus at room temperature, 3-diaminobenzidine tetrahydrochloride DAB (ab94665, Abcam, UK) was used to visualise specific antigen. Sections were counterstained with hematoxylin and washed with cool water. The number of nuclei with positive reactivity for Nrf2 and IL-17A were counted in the 3 areas of mucosal epithelium and lamina propria. The expression level of Nrf2 and IL-17A were assessed using a 6-score system (0 = negative, 0.5 = 0–5% positive, 1 = 5–15% positive, 2 = 16%–40% positive, 3 = 41%–90% positive, and 5 more than 90% positive).

2.4. Statistical analysis

All data were presented as mean ± SD and were evaluated by SPSS19.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). The distribution of data was normal and the relationship between age of subjects and groups was addressed with the Fischer exact test. Unpaired *t*-test was

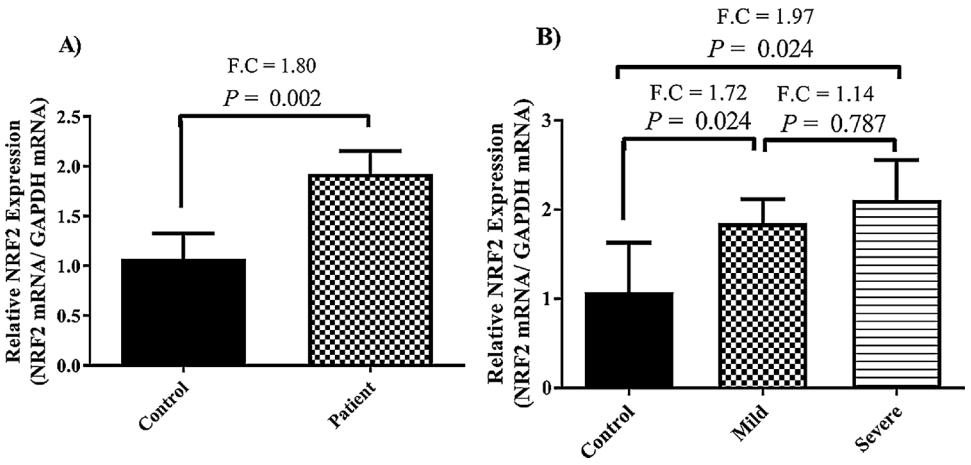

Fig. 1. A) Nrf2 mRNA level was evaluated in 20 UC and 21 control colonic tissues. Nrf2 gene expression in UC and control mucosa was shown. The data from the real-time PCR for human Nrf2 were normalized versus GAPDH. B) Nrf2 gene was significantly overexpressed in endoscopic specimens in mild and severe patients rather than control. UC: ulcerative colitis; F.C: fold change.

used to compare the 2 groups and multiple comparisons were done by Tukey post hoc. Pearson's correlation analysis was used to estimate the correlations between *Nrf2* gene expression and *GST-A4* and *PRDX1* gene expression. Differences calculating in *P*-values < 0.05 were considered to be statistically significant. A multivariate logistic regression model was used to assess correlation between gene expression levels and existence of ulcerative colitis.

3. Results

3.1. Increased expression of Nrf2 at inflammatory locations of IBD tissues

We firstly evaluated the gene expression of the Nrf2 in the colonic tissue of subjects with UC compared to control samples. As it was reported previously, Nrf2-P was increased in the colonic epithelium of UC [20]. Accordingly, we found a remarkable increase of *Nrf2* gene expression by real-time PCR in inflamed colonic tissues compared to control samples ($P = 0.002$, Fig. 1A). Moreover, mRNA level of Nrf2 in both mild and severe patients enhanced considerably; however, the comparison of *Nrf2* gene expression between severe and mild patients did not show any statistical signification ($P > 0.05$, Fig. 1B). The samples were immunostained with anti-Nrf2 antibody in order to characterise the locations of Nrf2 protein factor in the inflamed mucosa. Immunohistochemistry analysis indicated that intestinal epithelial cells (IEC) were a major source of Nrf2 in tissues, while lamina propria mononuclear cells appeared fewer Nrf2 staining. Interestingly, strong cytoplasmic staining of Nrf2 factor was detected among IECs in UC samples ($3 < \text{score} < 5$) compared with control samples ($2 < \text{score} < 3$) (Fig. 2A, C).

3.2. The different pattern of IL-17A and Nrf2 protein expression in inflammatory biopsies

As previous investigations showed that IL-17A was mostly expressed in the inflamed colonic tissue of UC patients. In contrast to the repetitive stain of Nrf2 in IECs, IL-17A was dominantly observed among immune cells ($3 < \text{score} < 5$) at mucosa layer (Fig. 2B). On the other hand, we found more labelling of IL-17A rather than Nrf2 staining at lamina propria regions (Fig. 2B). It means that, Nrf2 negatively correlated with IL-17A staining ($P = 0.004$, Fig. 3). In the normal mucosa, IL-17A was specific for mocusa regions, but it was not detected in lamina propria (Fig. 2D). IL-17A is less detected in intestinal inflammation presenting Nrf2 factor. Apparently, IL-17A staining was declined in the control samples rather than inflamed colonic tissue.

3.3. The mRNA expressions of GST-A4 and PRDX1 in ulcerative colitis

Real-time PCR was used to evaluate the mRNA expressions of GST-A4 and PRDX1 in the colonic samples of the control group and UC patients. The expression of GST-A4 mRNA in patients was two times higher than controls ($P < 0.001$); however, the comparison of *GST-A4* gene expression between severe and mild samples did not show any statistical signification (Fig. 4A and B). In addition, there was no significant difference in mRNA level of PRDX1 (Fig. 4C and D). As can be observed in the Table 2, the mRNA levels of GST-A4 and PRDX1 considerably correlated with that of in Nrf2. In multivariable logistic regression analysis, the existence of ulcerative colitis was significantly associated with high level of GST-A4 gene expression (95% CI, 3.602–4457.1, P = 0.008) (Table 3).

4. Discussion

The acquisition of inflammatory phenotypes in UC correlates with conserving signals derived from NRF2. Epithelial Nrf2 activation enhances intestinal wound healing and protects from apoptosis during oxidative injury [20,21]. The objective of this study was to recognize whether Nrf2 can be an efficient factor to arrest il-17 and promote GST-A4 and PRDX1 enzymes in UC patients. In addressing this question, the up-regulation of *Nrf2* gene expression was observed in our study, suggesting a possible preservation role for Nrf2 factor in the colonic tissue of UC patient. With regards to histological analysis, dominant presence of the Nrf2 factor in epithelial cells is likely to be an alternative in response to chronic inflammation. In a similar study, phosphorylated form of Nrf2, which is a nuclear type of this factor, was reported in IECs patients suffering from UC. However, elevated Nrf2 activity may give rise to the expression of proteasomal proteins resulting in colitis associated cancer process [20]. The active forms of Nrf2 factor constitutively protect epithelium integrity against effect of inflammatory cytokines in chronic mice colitis [5,7]. DSS-treated Nrf2-deficient mice exhibited colitis symptoms much earlier than WT mice [22]. Nevertheless, unstable Nrf2 proteins are not able to implement antioxidant defenses in chronic obstructive pulmonary disease (COPD) despite the high amount of this factor in bronchial cells [23]. Unstable Nrf2 factor in cytosol is usually tagged by the ubiquitin ligase adapter for proteasome degradation [24].

Cellular stress has been shown to induce inflammatory mediators in various cell systems in IBD [25]. Animal models indicated that oxidative stress is sufficient for the initiation of intestinal inflammation and extension to other organs [26]. Macrophage Nrf2 activation suppresses inflammatory cytokine by binding to the upstream region of M1-induced genes [27]. To investigate the interaction expression between IL-17A and Nrf2 involved in IBD mucosa, immunohistochemical stain of IL-17A and Nrf2 antibodies was used in colonic tissues. It seemed that mononuclear cells were more potent in inducing IL-17A in mucosa layer and lamina propria in comparison with the Nrf2 factor. It is speculated that production of IL-17A was irreversibly regulated by Nrf2, especially

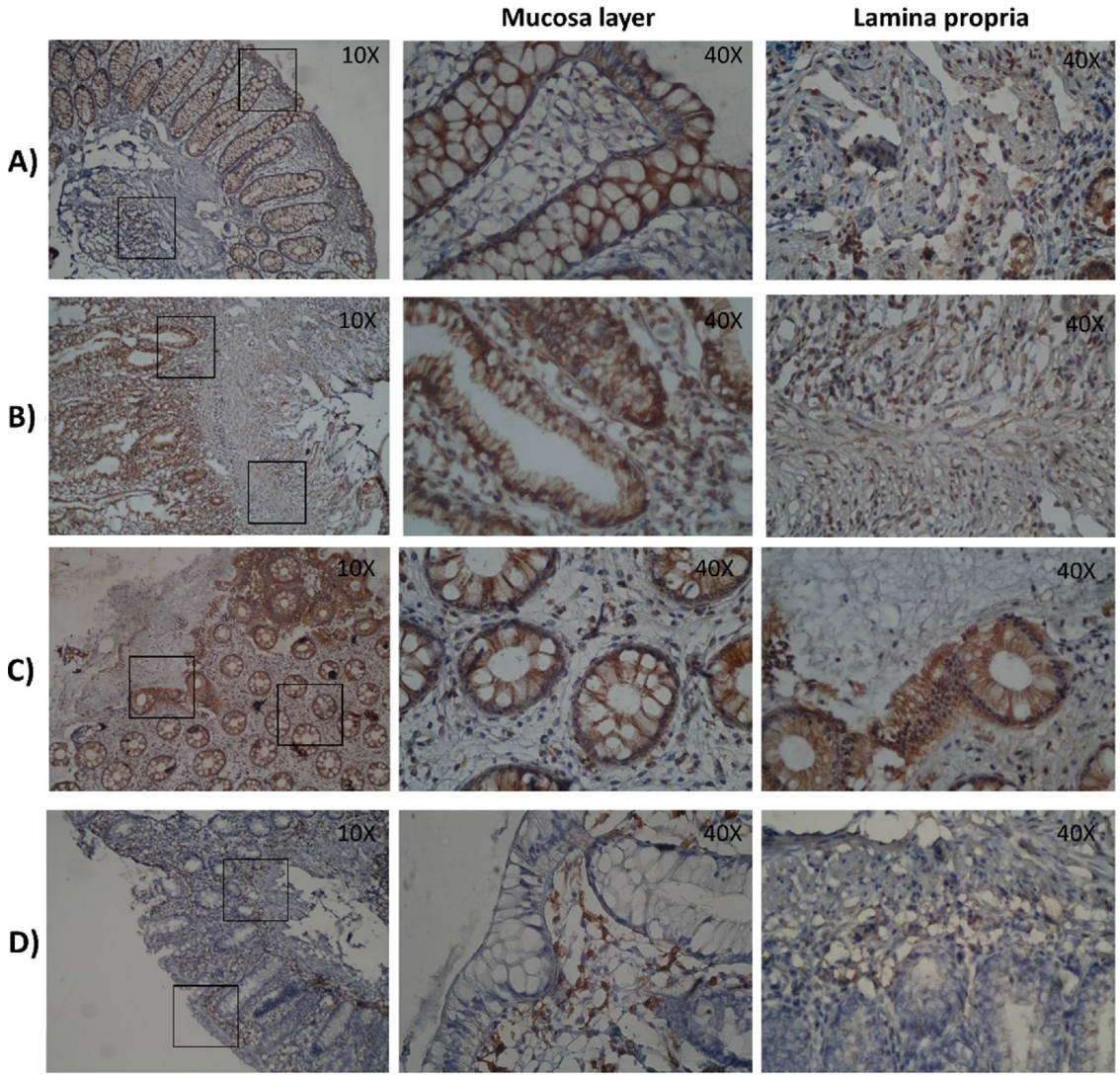

Fig. 2. Immunohistochemistry analysis of UC and normal colonic tissues for Nrf2 and IL-17A. Formalin-fixed and paraffin-embedded were stained with Nrf2 and IL-17A antibodies. A) IECs were a major source of Nrf2 in colonic UC biopsies compared to mononuclear cells. B) IL-17A has been stained in mononuclear cells in both mucosa and lamina propria regions of UC biopsies. C) Nrf2 in control biopsies dominantly appeared in IECs. D) On the contrary, IL-17A presented into mononuclear cells in control biopsies.

among epithelial and immune cells (Fig. 3). Activated Nrf2 is capable of inducing heme oxygenase-1 (Hmox-1) by which the IL-17 protein expression is reduced in mice with encephalomyelitis [8]. Likewise, Hmox-1 modulates IL-17 produced by T helper-17 in a mouse model of colitis [28]. In fact, IL-17 commonly has an inflammatory mediator that is activated by multitude cytokines of tissue-damaging in IBD. The induction of IL-17 is mediated by TNF-a, IL-1b, IL-6, GM-CSF, G-CSF. IL-17 is considered as a recruitment activation and migration for neutrophils. The retinoic orphan receptor gamma(t) (RORct) factor, a transcription factor of Th17 cell differentiation, is found to be expressed

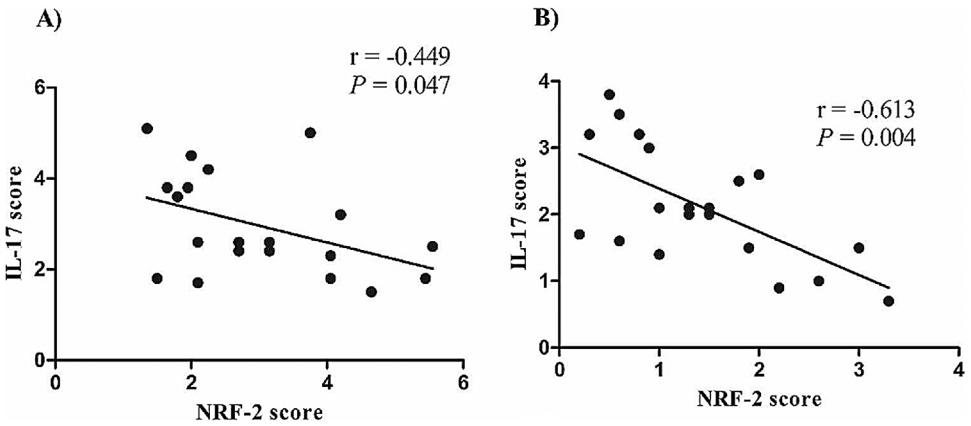

Fig. 3. The Immunohistochemistry data are presented for all patients diagnosed as the UC patients compared with control subjects. Staining scores from UC were correlated between IL-17A and Nrf2. The A) mucosa layer and B) lamina propria were analyzed based on a 6-score system (0 = negative, 0.5 < 5% positive, 1 < 15% positive, 2 < 16%–40% positive, 3 < 41%–90% positive, and 5 more than 90% positive).

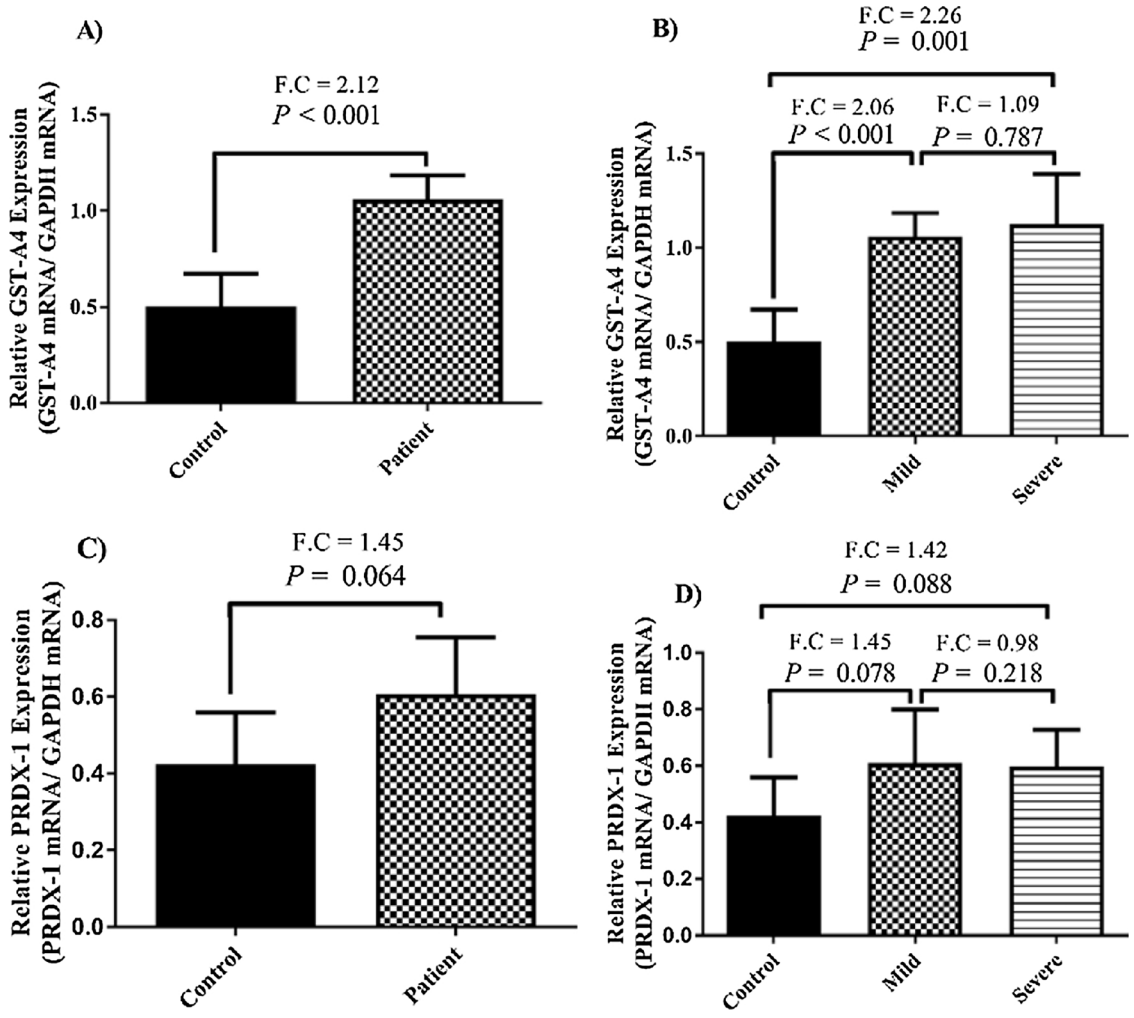

Fig. 4. The gene expressions of GSTA4 and PRDX1 were compared between control and patient group. The real-time PCR data were normalised versus GAPDH as the housekeeping gene. A considerable increase was observed in gene expression of GSTA4 in UC patients (both mild and severe state) compared to control group (A) and (B). We did not detect any significant difference in mRNA level of PRDX1 (C) and (D). P-values less than 0.05 is statistically significant.

Table 2
Pearson correlations for GSTA4 and PRDX1 with Nrf2.

Gene	Group			
	Control r (*P* - value)	Patient r (*P* - value)	Mild r (*P* - value)	Severe r (*P* - value)
GSTA4	0.624 (0.003)	0.763 (0.001)	0.809 (0.001)	0.637 (0.174)
PRDX1	0.596 (0.004)	0.564 (0.010)	0.642 (0.013)	0.397 (0.436)

Table 3
Coefficients of the logistic regression analysis for the existence of ulcerative colitis.

Gene	Regression coefficient (β)	Exp (β)	Standard error	significance	95% C.I for Eep (B)	
					Lower	Upper
Nrf2	−0.293	0.746	0.833	0.725	0.146	3.822
GSTA4	4.842	126.708	1.817	0.008[a]	3.602	4457.1
PRDX1	1.053	2.865	1.809	0.561	0.083	99.286

[a] The existence of ulcerative colitis was significantly associated with high level of GST-A4 gene expression.

particularly in lamina propria T cells of patients with Crohn's disease. IL-17A was also increased at inflamed mucosa tissues in addition to mononuclear cells in lamina propria regions of patients suffering from UC [10,29].

Oxidative stress has been implicated in the destruction of the colonic epithelium, and may cause damage to the DNA. Numerous investigations confirmed that mucosal damages are sufficient for the incidence of neoplasias [30,31]. To protect cell structure, antioxidant enzymes can be expressed when the Nrf2 transcription factor binds to the ARE sequence [12]. In this study, attempt was made to determine the relevant transcriptional processes of PRDX1 and GST-A4 induced by the Nrf2 transcription factor. The PRDX1 function with active-site cysteine suggests a specific chaperones role in the oxidative condition [32]. PRDX1 is clearly reduced upon loss of Nrf2 in human lung cancer [33]. Although *PRDX1* gene expression in our analysis had a positive correlation with that of Nrf2, the increase in the level of PRDX1 mRNA was not the same as Nrf2 mRNA in the patient group. On the other hand, PRDX1 did not seem to be efficient in protection from UC disease according to the outcomes of logistic regression. The PRDX1 enzyme was evident in the mitochondrial and cytoplasmic parts of intestinal epithelial cells in patients with UC. Therefore, the accumulation of PRDX1 enzymes in specific locations probably is a response to mucosal injury instead of elevating gene expression [34]. To the best of our knowledge, GST-A4 mRNA level was increased in both mild and severe state of the patient group. Besides the significant correlation between

GST-A4 and *Nrf2* gene expression, the exponential rate of logistic regression illustrated that GST-A4 may play a preservation role in inflamed colonic tissues (Table 3). Previous findings indicated that Nrf2 is accompanied with mitogen-activated protein kinase (MAPK) pathway resulting in up-regulation of *GST-A4* gene expression. Indeed, the c-Jun factor can induce GST-A4 after epithelial cells are exposed to ROS factors [16,35]. It is more likely that the activation of MAPK pathway collaborates to increase *GST-A4* gene expression in the patient group. Although these detoxification enzymes promote antitumor potent in UC disease, Nrf2 is identified as the master key regulator of actual carcinogenesis in inflammation-associated colorectal cancer [16,36].

In summary, we demonstrated that Nrf2 transcription factor was highly located in the epithelial cells of UC patients but IL-17A was identified more in immune cells rather than epithelial cells. One limitation of our analysis was that molecular interplay between the Nrf2 factor and IL-17A was not particularly applied in the *ex-vivo* study. Our data suggest that inflamed colonic cells under oxidative stress provoke Nrf2 transcription by which gene expression of GST-A4 and PRDX1 were elevated; however, PRDX1 gene expression is rather independent of Nrf2.

5. Conclusion

This study confirms an irreversible associations between the Nrf2 factor and IL-17A protein expression. Future investigations should focus on the molecular interaction between them, ideally using isolated IECs of UC patients or colonic cell line cultured in the inflammatory environment. There were no differences detected in gene expression of PRDX1. Future studies should also provide the opportunity to find the anti-oxidative stress performance in inflamed intestine, which would lead to a better understanding of this inflammatory disease.

Ethics approval and consent to participate

The study protocol was approved by the local medical ethics committee of Shahrekord University of Medical Sciences Ethics Committee with a number: IR.SKUMS.REC.1395.313.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due the principle of confidentiality of funding but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Disclosure statements

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Authors' contributions

Conceived and designed the experiments: MSG. Conducted the experiments: HS, MG. Analysed the data: FD. Contributed reagents/materials/analysis tools: MS, GR, AS. Wrote the paper: MSG, HS, MG. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.06.001.

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